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Short communication

# High throughput LC–MS/MS method for simultaneous determination of zidovudine, lamivudine and nevirapine in human plasma

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ABSTRACT

A selective and sensitive high performance liquid chromatography–tandem mass spectrometry method has been developed and validated for simultaneous determination of zidovudine (ZDV), lamivudine (3TC) and nevirapine (NVP) in human plasma. After Solid phase extraction (SPE), analytes and ISTDs were run on Peerless Basic C18 column with an injection volume of 3 μL and run time of 3.0 min. An isocratic mobile phase of 0.1% formic acid in water:methanol (15:85, v/v) was used with positive mass spectrometric detection. The method was validated over a concentration range of 5–1500 ng/mL for ZDV and 3TC and over the concentration range of 10–3000 ng/mL for NVP. The intraday and interday precision and accuracy across four validation runs were ranged from 1.6 to 10.1% and 93.8 to 110.8% respectively.



## 1. Introduction

Multi-class combination treatment has become the most common regime in the management of acquired immunodeficiency syndrome (AIDS) [1]. Due to increased resistance for the causative human immunodeficiency virus (HIV) [2], US Department of Health and Human services has recommended single and multiple class combination regimens which are often referred to as highly active antiretroviral therapy (HAART) [3]. The most common combination at the beginning of the treatment consists of two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI).

Zidovudine (ZDV) a nucleoside reverse transcriptase inhibitor acts as chain-terminator of viral DNA during reverse transcription [4]. Absorption of ZDV is rapid and nearly complete on oral administration [5], and because of first pass metabolism the systematic bioavailability of ZDV is approximately 65%. Lamivudine (3TC), another nucleoside reverse transcriptase inhibitor is active against HIV type-1 and hepatitis B (HBV) [6]. Phosphorylated active metabolites of 3TC competitively inhibit the HIV reverse transcriptase enzyme and act as a chain terminator of DNA synthesis. On oral administration 3TC absorption is rapid and absolute bioavailability is approximately 86% for both tablet and oral solution. Nevirapine (NVP) is a potent, non-nucleoside reverse transcriptase inhibitor used in combination with nucleoside analogs for HIV infection [7]. NVP binds directly to reverse transcriptase and blocks the RNA dependent and DNA dependent DNA polymerase activities by causing disruption in the enzymes catalytic site. On oral administration its systemic availability is about 90% with a relatively longer half life of 45 h.

Several analytical methods [8–16] have been developed for the determination ZDV, 3TC and NVP individually or in combination in pharmaceutical formulations, however very few methods are reported for simultaneous determination of ZDV, 3TC and NVP in biological matrices. Malm et al. [10] developed a combination method on blood samples using gradient HPLC with UV detection. The limit of quantification was 0.11, 0.13, and 1.3 μg/mL for 3TC, ZDV and NVP respectively. Vandana et al. [13] developed a gradient HPLC-UV method for the three drugs in plasma using 950 μL sample volume with a quantification limit of 51 ng/mL. Zhou et al. [14] developed an LC–MS/MS method for determination of ZDV, 3TC and NVP with a LOQ of 20 ng/mL using protein precipitation technique, which has a high potential for ion-suppression in LC–MS/MS analysis. Krishna et al. [15] developed a simultaneous LC–MS/MS method with a run time of 3.5 min and LOQ of 25 ng/mL for ZDV and 3TC and 81 ng/mL for NVP which is not sufficient for the current application.

Aim of the current study was to develop and validate [17,18] a more sensitive and selective high throughput LC–MS/MS method that can be efficiently used in pharmacokinetic studies, to evaluate bioavailability and bioequivalence for this potent combination

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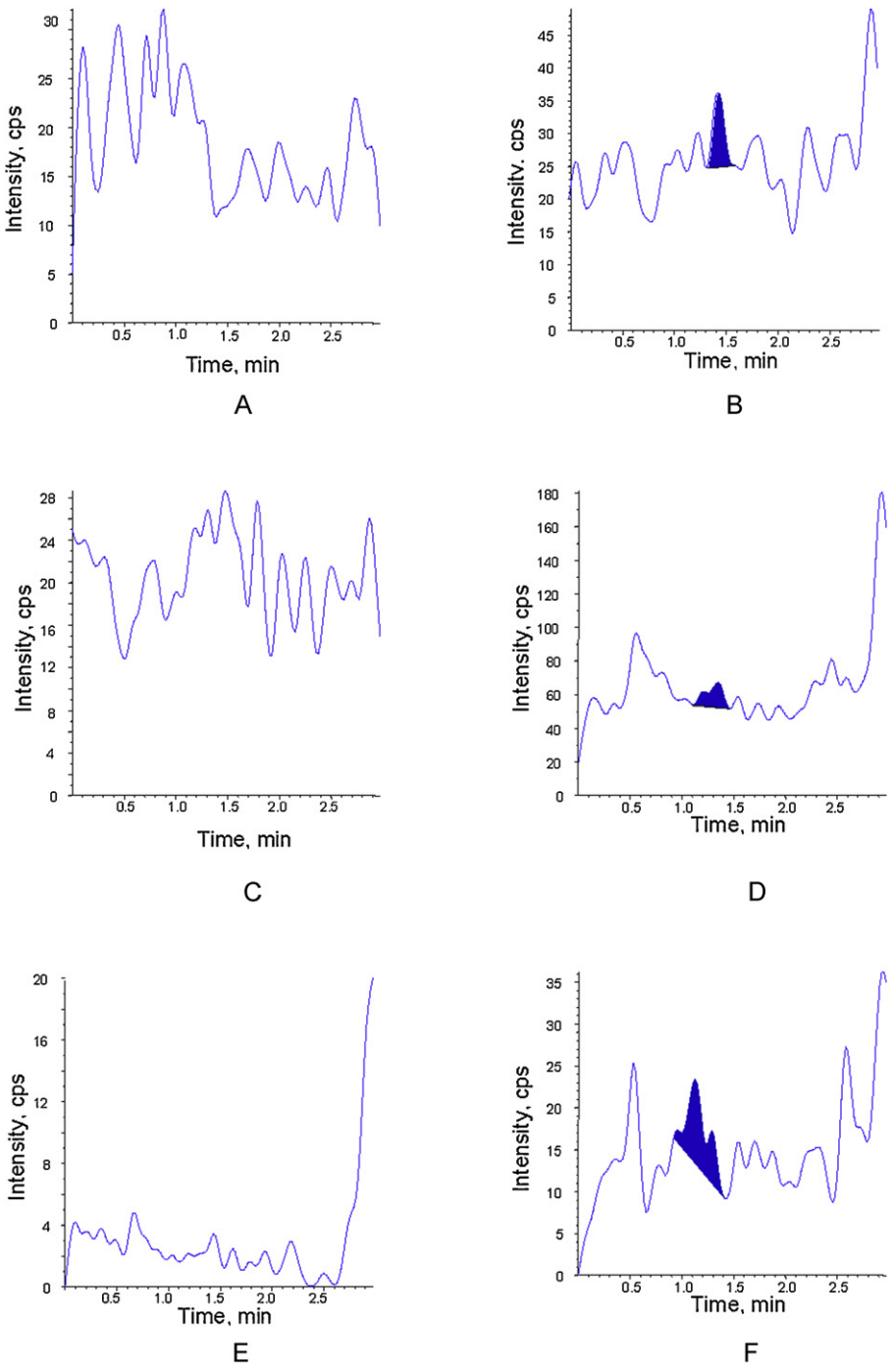


**Fig. 1.** Representative chromatograms of ZDV (A), DDI (B), 3TC (C), FTC (D), NVP (E) and ABC (F) in blank plasma.

of ZDV, 3TC and NVP. The new LC–MS/MS method was developed in human plasma containing $K_2$ EDTA as anticoagulant, and was completely validated as per FDA guidelines. A sensitive quantification limit of 5 ng/mL for ZDV and 3TC and 10 ng/mL for NVP was achieved with a sample processing volume of 300 μL.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Working standards of zidovudine (ZDV), lamivudine (3TC), nevirapine (NVP) and ISTDs having purity more than 99% were obtained from Aurobindo Pharma Limited (Hyderabad, India). Didanosine (DDI), emtricitabine (FTC) and abacavir sulfate (ABC) were used as ISTDs for ZDV, 3TC and NVP respectively. HPLC grade methanol and acetonitrile were procured from Thermo Fisher Scientific India Private Limited (Mumbai, India). HPLC Type 1 water obtained from a Milli-Q gradient system (Millipore, Bedford, USA) was used in analysis. Oasis HLB 30 mg; 1 CC SPE cartridges were purchased from Waters Corporation (Milford, USA).

### 2.2. Instrumentation

HPLC system (Shimadzu, Japan), equipped with LC-20AD pumps for solvent delivery, DGU-20 A3 degasser, CTO-AS vp Column oven and a high throughput a SIL HTc autosampler was used for the

analysis. Mass spectrometric detection was performed on an API-4000 triple quadrupole instrument (MDS-SCIEX, Canada) in multiple reaction monitoring (MRM) mode. A turbo ion spray interface in positive ionization mode was used and the data processing was performed on Analyst software, version 1.4.1 (SCIEX).

### 2.3. Chromatographic conditions

Chromatographic separation was performed on a Peerless Basic C18 column (100 mm × 4.6 mm i.d., 5 μm). An isocratic mobile phase comprising a mixture of methanol and 0.1% formic acid in water (85:15, v/v) was delivered at a flow rate of 0.8 mL/min. The column oven was maintained at 35 °C and the autosampler cooler temperature was set at 10 °C with an injection volume of 3 μL. The total chromatographic run time was 3 min.

### 2.4. Mass spectrometry conditions

Turbo ion spray interface (TIS) operated in positive ionization mode was used for the detection. The MRM transitions monitored were *m/z* 268.2 → 127.1 (ZDV), *m/z* 237.1 → 137.1 (DDI), *m/z* 230.2 → 112.1 (3TC), *m/z* 248.1 → 130.0 (FTC), *m/z* 267.2 → 226.1 (NVP) and *m/z* 287.2 → 191.2 (ABC) with a dwell time of 200 ms per transition and the quadrupoles 1 and 3 were set at unit resolution.

### 2.5. Preparation of calibration standards and quality control samples

Stock solutions of ZDV, 3TC, NVP and ISTDs were individually prepared at a concentration of 1 mg/mL in methanol. The prepared stock solutions were stored in refrigerator at below 10 °C and were brought to room temperature before use. Working solutions were prepared in methanol:water mixture (50:50, v/v) and are used in the assay.

Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with freshly prepared working solutions. Blank plasma lots obtained from healthy, non-smoking volunteers were individually screened and pooled before use. Calibration standards were made at concentrations of 5, 10, 25, 100, 400, 800, 1200, 1500 ng/mL for ZDV and 3TC; and 10, 20, 50, 200, 800, 1600, 2400, 3000 ng/mL for NVP.

Quality control samples were prepared at 5 ng/mL (LLOQ QC), 15 ng/mL (LQC), 800 ng/mL (MQC) and 1200 ng/mL (HQC) for ZDV and 3TC; and 10 ng/mL (LLOQ QC), 30 ng/mL (LQC), 1600 ng/mL (MQC) and 2400 ng/mL (HQC) for NVP. Aliquots of spiked plasma samples were taken in polypropylene tubes and were stored in freezer at −70 °C and −20 °C.

### 2.6. Sample preparation

A 300 μL aliquot of each plasma sample was transferred to a 5 mL polypropylene tube, followed by addition of 50 μL of ISTD solution (consisting of 2000 ng/mL DDI, 500 ng/mL FTC and 250 ng/mL of ABC) and 100 μL of 0.1% formic acid in water. The contents were briefly mixed by vortex and were subjected to solid phase extraction. Using positive pressure SPE unit, cartridges were conditioned with 1 mL each of methanol and Milli-Q water, followed by dispensing of plasma samples. Washing was performed with 1 mL each of Milli-Q water and methanol:water mixture (85:15, v/v). Finally the cartridges were eluted with 1 mL of acetonitrile and the eluates were evaporated to dryness under stream of nitrogen in a 50 °C water bath. The residue of each sample was reconstituted in 600 μL mobile phase and transferred to the chromatographic system.

## 3. Results and discussion

### 3.1. Method development

To have a consistent and reliable estimation of the analytes equal importance was given for optimizing the chromatographic conditions as well as extraction method. Analytes and ISTDs were tuned in positive ion mode using electro spray ionization technique. For NVP and its ISTD relatively high collision energy was applied to get optimum intensity. LC conditions were set under isocratic mode and gradient analysis was deliberately avoided without compromise in the run time and chromatography. Acidic mobile phase has improved the protonation of all compounds and addition of buffers even at 2 mM concentration has shown a negative impact on signal intensities. In the sample preparation protein precipitation and liquid-liquid extraction techniques were avoided due to high polar nature of 3TC and ZDV. Alternatively SPE technique with a basic protocol was employed, which resulted in consistent and reproducible recovery for all three analytes. During the optimization of extraction method, matrix effect was observed for 3TC and ZDV which was later eliminated by substitution of methanol with acetonitrile in the elution step.

### 3.2. Method validation

#### 3.2.1. Selectivity

Selectivity of the method in human $K_2$ EDTA plasma was evaluated in eight individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interferences were observed at the retention times of analytes and ISTDs.

**Table 1**
Intra batch and inter batch precision and accuracy.

| Analyte | QC Level | Nominal conc. (ng/mL) | Intra batch | | | Inter batch | | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean found conc.[a] (ng/mL) | %RE | %CV | Mean found conc.[b] (ng/mL) | %RE | %CV |
| Zidovudine | LLOQQC | 5.03 | 5.56 | 10.5 | 6.2 | 5.33 | 6.0 | 8.6 |
| | LQC | 14.79 | 15.10 | 2.1 | 2.3 | 14.74 | −0.3 | 6.5 |
| | MQC | 799.22 | 848.07 | 6.1 | 2.1 | 814.36 | 1.9 | 4.0 |
| | HQC | 1200.03 | 1329.62 | 10.8 | 2.7 | 1239.23 | 3.3 | 6.2 |
| Lamivudine | LLOQQC | 5.02 | 5.32 | 6.0 | 7.4 | 5.18 | 3.2 | 5.8 |
| | LQC | 14.77 | 14.80 | 0.2 | 1.8 | 14.68 | −0.6 | 3.9 |
| | MQC | 798.58 | 811.60 | 1.6 | 3.7 | 815.13 | 2.1 | 3.0 |
| | HQC | 1199.06 | 1234.46 | 3.0 | 3.8 | 1243.02 | 3.7 | 3.2 |
| Nevirapine | LLOQQC | 10.12 | 9.25 | −8.6 | 6.1 | 9.51 | −6.0 | 7.7 |
| | LQC | 29.64 | 28.73 | −3.1 | 1.8 | 29.16 | −1.6 | 5.1 |
| | MQC | 1600.26 | 1631.14 | 1.9 | 3.8 | 1659.85 | 3.7 | 2.0 |
| | HQC | 2402.82 | 2365.53 | −1.6 | 3.8 | 2396.09 | −0.3 | 2.3 |

%CV – percent coefficient of variation; %RE – percent relative error.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 24 replicates at each concentration.

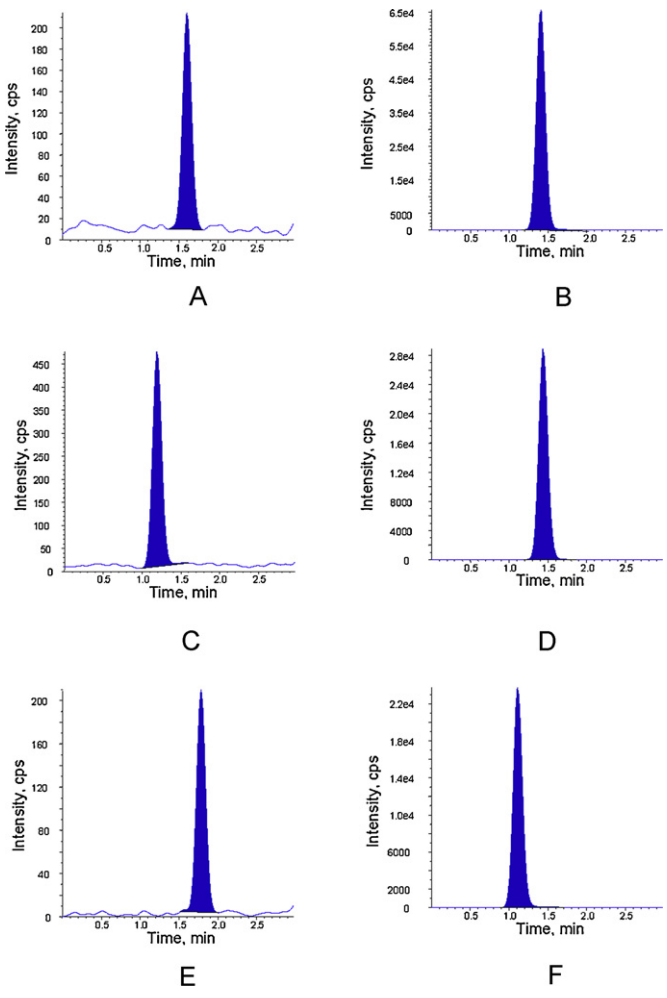


**Fig. 2.** Representative chromatograms of ZDV (A), DDI (B), 3TC (C), FTC (D), NVP (E) and ABC (F) in LLOQ sample.

Figs. 1 and 2 demonstrate the selectivity of the method, with the chromatograms of blank plasma and LLOQ sample respectively.

### 3.2.2. Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak-area ratio ($y$) of analytes to ISTDs versus the nominal concentration ($x$) of analytes. Calibration curves were linear with coefficient of correlation ($r^2$) values more than 0.9926. The $r^2$ values, slopes and intercepts were calculated using weighted ($1/X^2$) linear regression analysis with four intra and inter day calibration curves. At LLOQ, accuracy (%RE) for all the three analytes was ranged from −6.0 to 6.0%, with a %CV of <8.6%. The mean signal to noise ratio at LLOQ ($n = 6$) was found to be 150:1, 123:1 and 118:1 for 3TC, ZDV and NVP respectively.

### 3.2.3. Precision and accuracy

Precision and accuracy was evaluated using four intra and inter day precision and accuracy runs, with each batch consisting of six replicates of quality control samples at four concentration levels (LLOQ, LQC, MQC and HQC). The intra and inter batch precision was less than 8.6% for ZDV, 3TC and NVP with accuracy (%RE) between −8.6 and 10.8. Results of precision and accuracy were presented in Table 1.

### 3.2.4. Matrix effect

Matrix effect was investigated by extracting blank plasma from six different sources, including one hemolytic and one lipemic lot. After extraction, residue from each lot was reconstituted with mobile phase having known amount of analyte (LQC level along with ISTD; post extracted samples) and analyzed along with equivalent aqueous samples. Matrix related ion suppression or enhancement was evaluated by calculating the IS normalized matrix factor. The mean IS normalized matrix factor for all the three analytes was ranged from 0.93 to 1.02 with a %CV of ≤8.5.

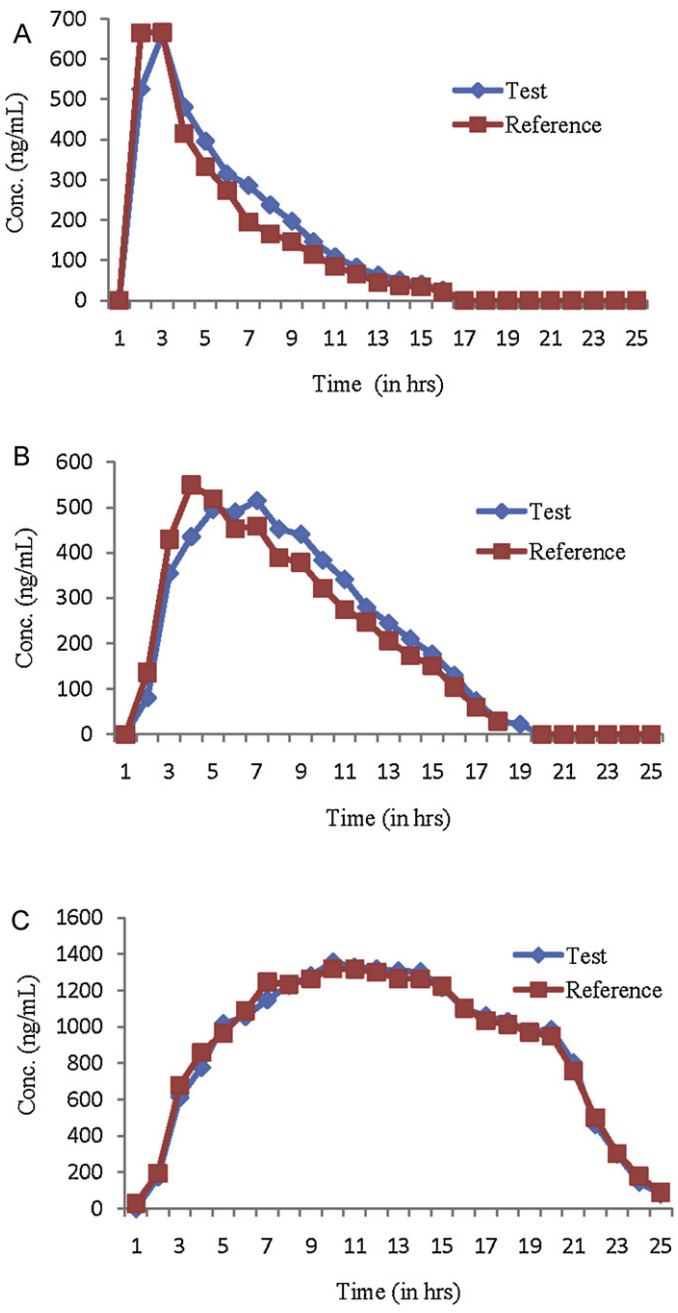


**Fig. 3.** Mean plasma concentration versus time profile of ZDV (120 mg) (A), 3TC (60 mg) (B) and NVP (100 mg) (C) after oral administration to 36 healthy Indian male volunteers under fasting condition.

### 3.2.5. Extraction recovery and dilution integrity

The extraction recovery of analytes was determined by comparing peak areas from plasma samples ($n = 6$) spiked before extraction with those from plasma samples spiked post extraction. The mean recovery of ZDV, 3TC and NVP was found to be 77.6%, 50.2%

**Table 2**
Stability results for zidovudine, lamivudine and nevirapine.

| Stability | Analyte | QC Level | A | %CV | B | %CV | % Stability |
|---|---|---|---|---|---|---|---|
| Bench-top (18 h at ~25 °C) | Zidovudine | LQC | 14.67 | 6.9 | 14.86 | 6.9 | 98.7 |
| | | HQC | 1207.82 | 6.2 | 1241.99 | 7.2 | 97.2 |
| | Lamivudine | LQC | 14.93 | 8.4 | 14.79 | 4.8 | 100.9 |
| | | HQC | 1233.59 | 3.1 | 1221.06 | 2.7 | 101.0 |
| | Nevirapine | LQC | 30.57 | 2.8 | 28.95 | 5.1 | 105.6 |
| | | HQC | 2414.42 | 1.5 | 2397.83 | 4.0 | 100.7 |
| Freeze-thaw (after 4th cycle) | Zidovudine | LQC | 15.66 | 8.4 | 14.86 | 6.9 | 105.4 |
| | | HQC | 1221.48 | 2.9 | 1241.99 | 7.2 | 98.3 |
| | Lamivudine | LQC | 15.07 | 4.2 | 14.79 | 4.8 | 101.9 |
| | | HQC | 1221.77 | 3.3 | 1221.06 | 2.7 | 100.1 |
| | Nevirapine | LQC | 30.02 | 3.5 | 28.95 | 5.1 | 103.7 |
| | | HQC | 2399.55 | 3.0 | 2397.83 | 4.0 | 100.1 |
| In-injector (at 10 °C for 41 h) | Zidovudine | LQC | 15.38 | 8.2 | 15.43 | 8.2 | 99.7 |
| | | HQC | 1269.39 | 6.5 | 1285.20 | 5.3 | 98.8 |
| | Lamivudine | LQC | 14.57 | 7.6 | 15.09 | 8.2 | 96.6 |
| | | HQC | 1191.51 | 2.7 | 1195.45 | 3.0 | 99.7 |
| | Nevirapine | LQC | 30.12 | 5.7 | 29.65 | 5.1 | 101.6 |
| | | HQC | 2411.63 | 2.3 | 2406.67 | 3.9 | 100.2 |
| Dry extract (at 1–10 °C for 41 h) | Zidovudine | LQC | 15.21 | 3.8 | 15.43 | 8.2 | 98.6 |
| | | HQC | 1245.18 | 6.1 | 1285.20 | 5.3 | 96.9 |
| | Lamivudine | LQC | 15.42 | 6.3 | 15.09 | 8.2 | 102.2 |
| | | HQC | 1215.31 | 2.4 | 1195.45 | 3.0 | 101.7 |
| | Nevirapine | LQC | 30.93 | 4.0 | 29.65 | 5.1 | 104.3 |
| | | HQC | 2376.97 | 2.1 | 2406.67 | 3.9 | 98.8 |
| Long-term stability (at −70 °C for 75 days) | Zidovudine | LQC | 15.03 | 4.0 | 15.06 | 6.7 | 99.8 |
| | | HQC | 1166.80 | 4.4 | 1136.71 | 5.4 | 102.6 |
| | Lamivudine | LQC | 13.99 | 3.0 | 13.86 | 5.9 | 100.9 |
| | | HQC | 1205.94 | 0.5 | 1214.97 | 5.4 | 99.3 |
| | Nevirapine | LQC | 30.86 | 5.7 | 31.37 | 5.0 | 98.4 |
| | | HQC | 2363.61 | 2.5 | 2500.65 | 2.0 | 94.5 |
| Long-term stability (at −20 °C for 75 days) | Zidovudine | LQC | 15.60 | 2.2 | 15.06 | 6.7 | 103.6 |
| | | HQC | 1135.32 | 4.8 | 1136.71 | 5.4 | 99.9 |
| | Lamivudine | LQC | 13.83 | 6.7 | 13.86 | 5.9 | 99.8 |
| | | HQC | 1188.06 | 3.2 | 1214.97 | 5.4 | 97.8 |
| | Nevirapine | LQC | 30.19 | 4.9 | 31.37 | 5.0 | 96.2 |
| | | HQC | 2431.38 | 2.3 | 2500.65 | 2.0 | 97.2 |

A – mean concentration (ng/mL) of stability samples; B – mean concentration (ng/mL) of comparison samples.

and 78.5% respectively with %CV across the three levels ranging between 1.2 and 13.9%.

Dilution integrity of the method was evaluated after ½ and ¼ dilution. The mean back calculated concentrations for ½ and ¼ dilution samples were within 85–115% with a %CV of ≤3.6.

### 3.2.6. Stability

Stability evaluations were performed in both aqueous and matrix based samples. The stock solutions were stable for a period of 22 h at room temperature and up to 7 days at 1–10 °C. The stock dilutions in methanol:water mixture (50:50, v/v), were stable up to 20.0 h at room temperature.

Stability evaluations in matrix were made using freshly spiked calibration standards and quality control samples (comparison samples). Analytes were stable up to 18 h on bench top and over 4 freeze thaw cycles. The processed samples were stable up to 41 h in autosampler at 10 °C and the dry extract samples were stable up to 41 h at 1–10 °C. The long-term matrix stability was evaluated at both −20 °C and −70 °C over a period of 75 days. No significant degradation of analytes was observed over the stability duration and conditions. Results of the stability evaluations were presented in Table 2.

### 3.3. Application

The validated method was successfully applied to 'An open label, randomized, two, treatment, two sequence, two period, cross-over, single-dose comparative oral bioavailability study of fixed dose combination of ZDV, 3TC and NVP tablets for oral suspension (TFOS, 2 × 60/30/50 mg, Test formulation) and Retrovir syrup (50 mg/5 mL of ZDV, 120 mg dose, Reference), Epivir oral solution (10 mg/mL of 3TC, 60 mg dose, Reference), Viramune oral suspension (50 mg/5 mL of NVP, 100 mg dose, Reference)', conducted over 36 healthy male human volunteers. The precision and accuracy of calibration standards and quality control samples analyzed along with study samples were within the acceptance limits and the mean plasma concentration versus time profiles for ZDV, 3TC and NVP under fasted condition are presented in Fig. 3.

## 4. Conclusion

A rapid, sensitive and accurate liquid chromatography with tandem mass spectrometry method was developed and validated for simultaneous determination of ZDV, 3TC and NVP in human plasma with a low run time of 3.0 min. The method offers high sensitivity and throughput over the already reported methods [14,15] with a LOQ of 5 ng/mL for ZDV and 3TC and 10 ng/mL for NVP. The extraction method utilizes a more selective solid phase extraction technique, offering consistent and reproducible recoveries for all three analytes with insignificant interference and matrix effect. The analytes are stable under the proposed conditions and the established LOQ is sufficiently low to conduct a pharmacokinetic study with fixed dose formulation containing [2 × 60 (ZDV) + 30 (3TC) + 50 (NVP)] mg.

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